Faculty of Science

# AN ACE IN THE HOLE: EXPLOSIVE SEED DISCHARGE BY *ARCEUTHOBIUM AMERICANUM* (LODGEPOLE PINE DWARF MISTLETOE) MAY BE FACILITATED BY DECLINING STOMATAL DENSITY

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**B.Sc. Honours thesis**

THOMPSON RIVERS UNIVERSITY

# AN ACE IN THE HOLE:

# EXPLOSIVE SEED DISCHARGE BY *ARCEUTHOBIUM AMERICANUM* (LODGEPOLE PINE DWARF MISTLETOE) MAY BE FACILITATED BY DECLINING STOMATAL DENSITY

by

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## ABSTRACT

*Arceuthobium americanum*, the lodgepole pine dwarf mistletoe, is a dioecious parasitic flowering plant that infects lodgepole pine (*Pinus contorta* var. *latifolia*) in the Pacific Northwest. The infection causes stunted growth of the host tree while also compromising timber value. The plant utilizes a unique dispersal strategy involving explosive seed discharge, and thus understanding its reproductive biology is an integral step toward managing its spread. *A. americanum's* complete life cycle occurs over five to six years, and the fruit matures over two consecutive growing seasons. Of particular interest are stomata (small pores in the plant's epidermis), which not only permit gas exchange, but also allow for cooling through transpiration. The primary goal of this study was to observe changes in fruit development and morphology using environmental scanning electron microscopy (SEM). Developmental changes in second-year fruits were assessed to gain a better understanding of the underlying physiological processes and changes that precede explosive discharge. The length and diameter of second-year fruits were found to significantly increase over the growing season (April-September), whereas stomatal density significantly decreases over the same time period. Developmentally, the fruit was observed to swell, and the floral organs persisted through the season. The decline in stomatal density may be simply a consequence of the expansion of surface area, but could function in retaining water inside the fruit to facilitate discharge and/or provide a heating mechanism through reduced transpiration. Future work should explore the influence of stomata in the explosive discharge directly, by measuring discrete transpiration levels.

Thesis Supervisor: Professor (Full) Cynthia Ross Friedman, Ph.D.

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# INTRODUCTION

*Arceuthobium americanum* (lodgepole pine dwarf mistletoe) is a forest pest in western North America. All members of the flowering plant genus *Arceuthobium*, collectively known as dwarf mistletoes (DM), are dioecious. *A. americanum* parasitizes *Pinus contorta* var. *latifolia* (lodgepole pine) in British Columbia and impacts the forest industry by reducing timber quality and availability (Hawksworth and Wiens 1996). DM infection reduces starch abundance within the host needles (Chhikara and Ross Friedman 2008) and stunts growth (Logan et al. 2013). Annual timber losses from *A. americanum* are estimated at 3.8 million $m^3$ in western Canada and 11.3 million $m^3$ in the western United States in 2003 (Shamoun et al. 2003).

DM undergoes a five- to six-year life cycle consisting of host inoculation, maturation of the internal haustorial or "endophytic" system (the "roots" of the DM), and development of the vegetative plant external to the host (Hawksworth and Wiens 1996). In the complete life cycle, explosively-dispersed seeds germinate on a viable host and penetrate the wood to invade the vasculature via a haustorium. The majority of the plant's total mass is within the wood as the endophytic system, and after three to four years, the vegetative plant emerges from the host (Brandt 2006). *A. americanum* possesses highly reduced vegetative features, having fused whorls of leaves modified into a bract-like cupule that subtends the pedicels and stems. Highly-reduced flowers appear a year or two following shoot development. While the male plant's flowers mature and spread pollen in one season, the female flower appears and persists for two years after its inception. The female flowers are epigynous, consisting of two fused tepals that clasp the style at the distal end of the flower, exposing the stigma. The ovary is inferior and after fertilization, matures into a pseudoberry. The flowers also do not possess showy features for attracting pollinators. During its first year, the female flower becomes fertilized; the second year of fruit development is then dedicated to physiological preparation for seed dispersal.

To spread its seeds, *A. americanum* utilizes explosive seed discharge, which is facilitated by a variety of processes, only some of which are understood. Since discharge occurs in the plant's second year, morphological analysis of the second-year fruit will increase our knowledge of how this process occurs. Examination of fruit tissue development preceding the discharge has shown that the mesocarp differentiates into two distinct layers: the vesicular layer and the viscin

layer (Kelly et al. 2009). The outer vesicular layer consists of cells rich in lipid bodies, whereas the viscin layer is made up of elongate cells that secrete a mucilaginous liquid to form the viscin matrix. These layers are believed to function in the build-up of pressure via the accumulation of water (viscin) and the prevention of water loss (vesicular cells). Thermogenesis has been discovered in the plant, and also been shown to be integral to its discharge process (deBruyn et al. 2015). As the fruit matures in the second year, it recurves downwards to optimize the dispersal distance away from the parent plant. The seed is shot from the proximal end of the fruit at an angle of 45°, and travels in an arc that can carry it up to 16 m (in horizontal distance) from the parent plant (Hawksworth and Wiens 1996). The internal development of the male flowers has been documented as well, with the authors noting the unique anther wall formation in the *Arceuthobium* genus (Munro et al. 2014).

DM reproduction, including fruit growth and seed discharge, is critical to its ability to parasitize hosts, but there are few studies examining DM fruits and the details of their morphological development. To assess external female morphological development in *A. americanum,* attention should be given to the stomatal density, determining if the density changes over development. Stomata are small openings in the plant epidermis most commonly found on leaves, but also found on other organs, such as flowers and fruit. Stomata function in gas exchange for use in photosynthesis; however, *A. americanum* has very limited photosynthetic activity at maturity (Hull and Leonard 1964), and thus their stomata do not function in primarily in $CO_2$ uptake and $O_2$ venting. Stomatal density is of particular interest considering that DM performs little to no photosynthesis (Hull and Leonard 1964). Transpiration through the stomata draws water from the host to the parasite and is therefore a key component of DM physiology (Kirkpatrick 1989); thus, as stomata are fully implicated in the transpirational losses and water movement, a better understanding of stomata in DM is needed. Additionally, as *A. americanum*'s photosynthetic activity is highly limited and instead draws its organic carbon from the host, its cellular respiration produces ATP from the photosynthate received from the host. In other words, the stomata must be open to receive oxygen from the air so that the DM can create energy by respiration. As a result, stomatal density may directly affect growth of the DM, permitting the synthesis of energy once the photosynthate has been shuttled from the host into the DM.

Mature DM fruit are said to possess transverse stomata (Hawksworth and Wiens 1996), which are rare among the anthophyta (Butterfass 1987), but there is no literature detailing how these stomata are organized or how they develop. Due to the fact that at maturity, DMs are dependent on their hosts for photosynthate (Hull and Leonard 1964), stomatal density may be altered in response to processes other than photosynthesis. Water movement via transpiration is another integral function of stomata, functioning in temperature regulation as well as movement of minerals through the plant (Tanner and Beevers 2001). If substantial changes in stomatal density do occur, such changes would suggest a profound impact on *A. americanum's* physiology. Additionally, the large volume of water supplied by the tree should theoretically be more than enough to hydrate the DM, which in turn would reduce or eliminate the DM's need to close its stomata.

## Purpose

The purpose of this work was to observe and quantitatively measure changes in the *A. americanum* fruit during its second year of maturation. Use of scanning electron microscopy allowed for a better understanding of the morphological changes that precede discharge. Fruit length/diameter and stomatal density was measured over the growing season (April-September) of 2015 and morphological changes that occurred during maturation were noted. The characteristics of mature seeds were also observed. With this knowledge, we intended to expand our understanding of the physical changes and characteristics that the DM fruit undergoes prior to explosive discharge, and thus our understanding of *A. americanum* seed dispersal. I aimed to 1) determine if both the tepals and stigma persist through the second year of growth, 2) identify trends in fruit length/diameter and stomatal density, and 3) infer what the observed changes mean for the development of the plant on a physiological and cellular level. In doing so, future methods to control this forest parasite can more accurately target its dispersal mechanism.

# MATERIALS AND METHODS

## Collection

Female *A. americanum* were sampled from a previously sampled lodgepole pine (*Pinus contorta* var. *latifolia*) research site in close proximity to Stake Lake, south of Kamloops, British Columbia, Canada (50° 31′ latitude and 120° 28′ longitude). For this study, five trees were selected based on having a suitable amount of DM and were marked with flagging tape. From these trees, female *A. americanum* fruits growing 1-2.5 m from the ground were sampled weekly from April to September in 2015.

## Microscopy and Image Analysis

Upon return to the university (about 30 minutes from the time of collection), each female *A. americanum* sample was examined immediately with a scanning electron microscope (SEM; the Zeiss LS Evo). The samples were placed on double sided carbon tape and then put on aluminum stubs. No further preparation of the samples was needed, as the microscope used is an environmental SEM. The Zeiss LS Evo SEM is capable of sustaining a partial pressure of air to diffuse excess charge that accumulates on living samples when exposed to the electron beam. The specimens were placed in a partial vacuum at an extended pressure of 56 Pa (Dr. Cynthia Ross Friedman (pers. comm. Jan 2015). During this examination, general morphological observations were recorded and stomatal density on the fruits was determined using image analysis freeware (ImageJ); I used techniques similar to those employed in a 2008 study assessing pine needle health of infected and healthy hosts (Littley et al. 2008). To promote seed discharge, the mature fruits were heated in Ziploc™ bags (to about 30°C) to induce discharge and the recovered seeds were placed in the SEM. Stomatal density was determined by counting the stomata and dividing the total by the calculated surface area of the fruit. The surface area was calculated by treating the flower at the apex of the fruit as a cone and using ImageJ to measure the surface area. To measure the cone, a triangle was drawn to fit the flower in Microsoft Powerpoint, and the resulting image was imported into ImageJ, where the dimensions of the triangle were measured to determine the surface area of the cone. Stomata were counted on one side of the fruit from the SEM micrographs and doubled to estimate the total number of

stomata. Fruit growth was monitored by measuring the diameter (at the widest point) and length (from the base of the fruit to the tip of the stigma) of the fruits using ImageJ freeware.

## Data Analysis

This study was both qualitative and quantitative. Qualitatively, the overall patterns of female morphological development were charted through careful documentation with an SEM. Quantitatively, the stomatal densities, and fruit length/diameter of second year dwarf mistletoe fruit samples were recorded once per week, beginning in April 2015 and ending in September 2015. Dates were converted to ordinal values ("day-of-year") using a perpetual Julian date calendar (non-leap year) for use as a predictor variable in regression analyses. The response variables (fruit length/diameter and stomatal density) among the trees sampled were averaged for each date. The relationships between day of year and (1) fruit length and (2) fruit diameter were examined using simple linear regression. Polynomial regression was used to assess the relationship between stomatal density and day of year. Sample sizes were variable among each date and the standard error was calculated for each.

# RESULTS

## Developmental Observations

The SEM was used to observe developmental changes in second year DM fruit through the growing season. Figure 1 illustrates the morphology of a DM fruit, showing the floral structures at the apex of the fruit; that is, the tepal whorl and the stigma.

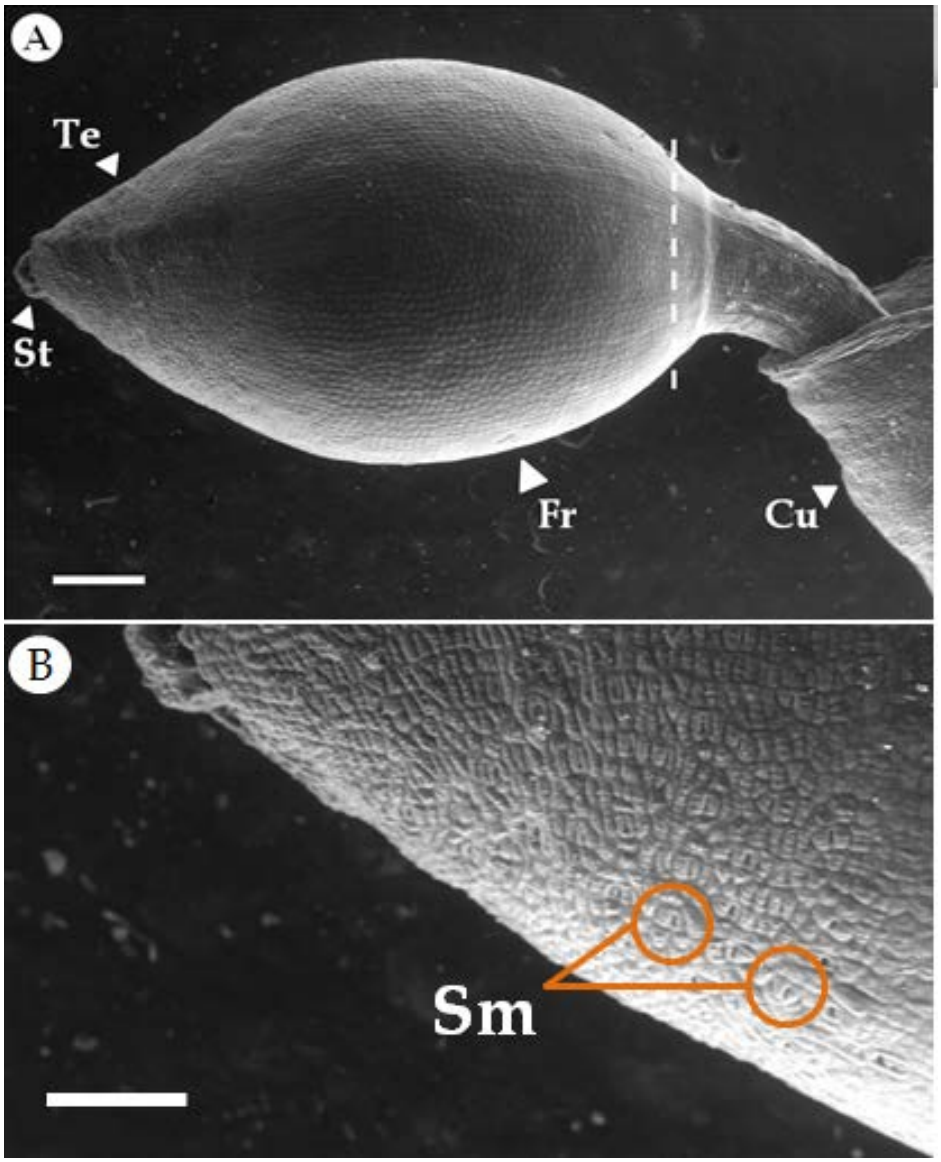


**Figure 1**. Morphology of the second year DM fruit, showing **(A)** the two fused tepals, forming the tepal whorl (Te), the stigma (St), the cupule (Cu), and the fruit (Fr). Dashed line indicates the abscission layer that fractures during discharge. Stomata (Sm) are visualized in **(B)**, showing transverse orientation. Micrographs taken on July 7, 2015. Bar represents 400 µm (A) and 200 µm (B).

The tepal whorl consists of two fused tepals surrounding the protruding stigma (Figure 1). This whorl possesses visible transverse stomata (Figure 1B), but the fruit body was never observed to have stomata at any time over the course of this study. The pedicel and the bract that subtend the floral organs possessed stomata perpetually through April-August 2015. The abscission layer that severs after explosive discharge was found to be located close to the pedicel at the proximal end of the fruit, approximately 80 µm from where the pedicel joins the fruit.

As would be expected, DM fruit grows in size and changes position on the plant (Figure 2). Stomatal density was determined by counting all visible stomata on the epidermis of the flower and dividing it by the calculated area of the tepal whorl. All the stomata seen on the tepal whorl were counted, as stomata may close as an artefact of the SEM's high vacuum, which dehydrates living samples.

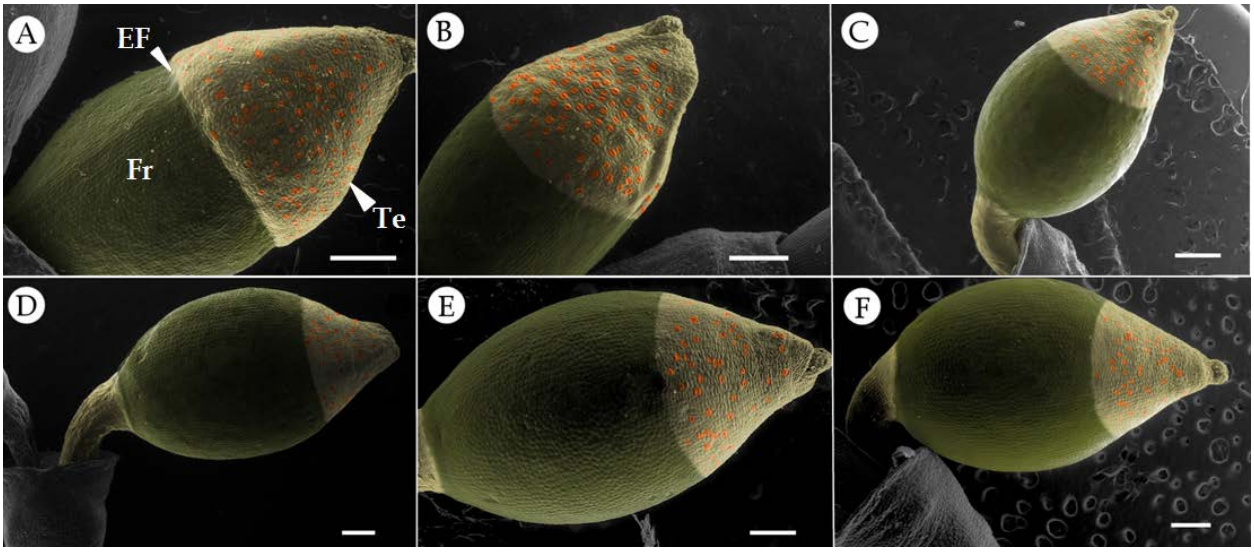


**Figure 2**. Second year DM fruit SEM micrographs taken in 2015 on **(A)** April 29 **(B)** May 28 **(C)** June 30 **(D)** July 28 **(E)** August 25 and **(F)** September 1. Images have been artificially colourized using Photoshop CS5.1 Extended and GIMP 2, so that the fruit (Fr) is highlighted in green, the tepal whorl (Te) in beige, stomata in orange, and the epidermal fold (EF) where the tepal whorl meets the fruit. Development over time visually indicates an expansion in diameter and an increase in length with a progressively declining stomatal density. Tepals and stigma/style persist through the entirety of the growing season. Scale bars in all images represent 400 µm.

Changes in the development of the DM fruit over time were imaged and colourized for comparison (Figure 2). In Figure 2A and Figure 2B, the tepal whorl diameter is comparable to that of the fruit diameter: 1.5 mm and 1.4 mm, respectively. In Figure 2A, the tepal whorl meets

the fruit tissue with a visible ring created by an epidermal fold (EF). The EF was present in all early fruits imaged, but disappeared as the fruit expanded, as is evident in Figure 2B onward to Figure 2F. As the fruit develops, its diameter expands so that it becomes visibly larger than the tepal whorl. By September (Figure 2F), the tepal whorl diameter had changed very slightly to 1.6 mm, a 14% increase, but the fruit diameter had expanded to 3.2 mm, a 113% increase since April. The tepal whorl and the stigma of the flower persist into the second year of development despite fertilization occurring the year prior. The fruit position relative to the cupule also changes over the season, gradually coming to point downward (Figure 3).

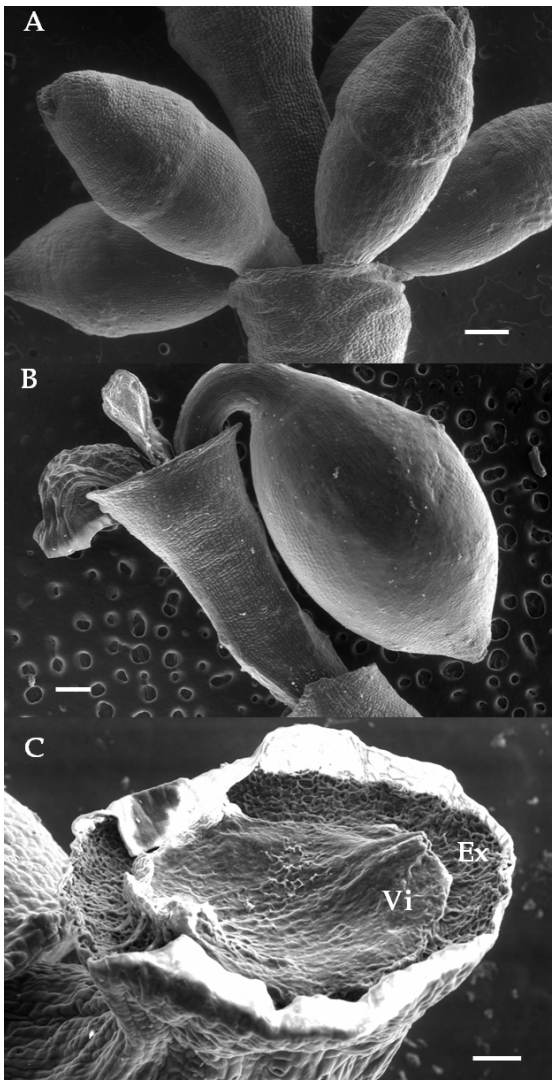


**Figure 3**. *A. americanum* fruit position on **(A)** April 29, 2015 (upright) and **(B)** August 18, 2015 (recurved). After discharge, fruits are severed from the parent at the abscission layer **(C)**, leaving only the base of the fruit attached to the pedicel. The discharged seed leaves behind a small amount of viscin (Vi) encircled by the exocarp (Ex). Bars represent 400 µm (A-B), and 100 µm (C).

Fruit orientation on the plant also changes as development progresses (Figure 3). At the beginning of the growing season, the fruit is erect/sits upright, emerging from the bract that subtends the inflorescences (Figure 3A). Approaching maturity, *A. americanum* fruits recurve downward (Figure 3B). While the pedicel and the small adjoining section of the base of the fruit remains attached to the plant post-discharge, the fruit and the tepal whorl is completely disconnected and drops from the parent. A small amount of viscin matrix is retained on the base of the fruit post-discharge (Figure 3C).

As the *A. americanum* fruit matured, a discernible cuticle was noted to develop over the epidermis of the fruit, but not the tepal whorl. This phenomenon was noted when the cuticle of a sampled fruit fractured in the SEM (Figure 4).

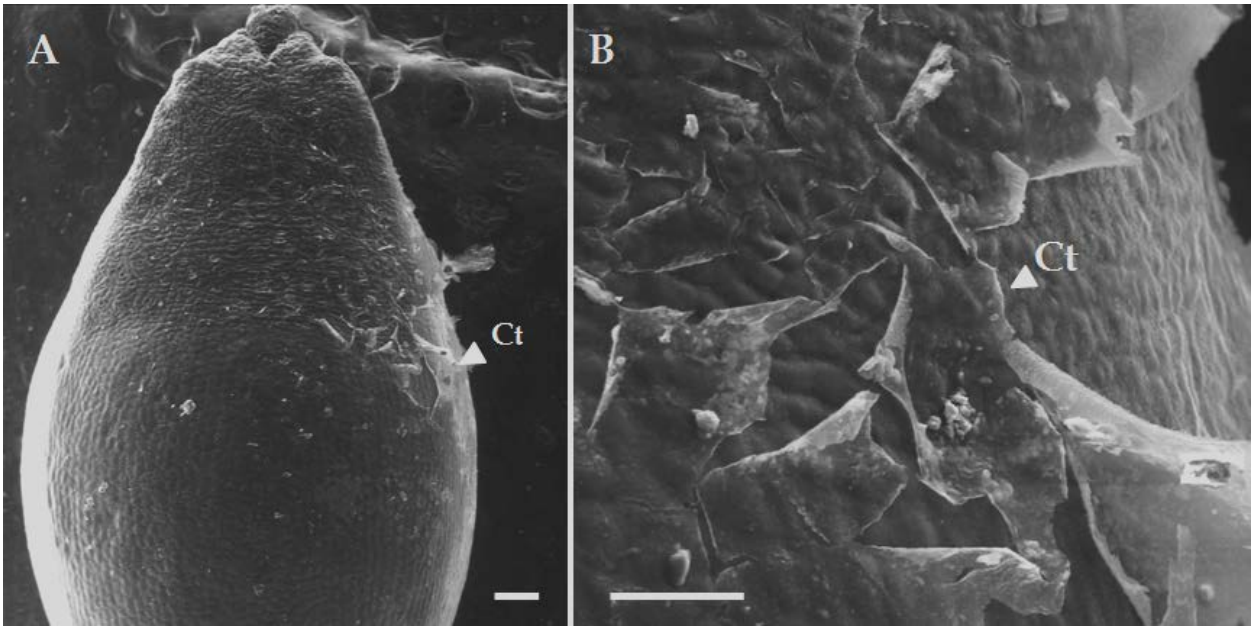


**Figure 4.** Fractured cuticle (Ct) of *A. americanum* fruit from August 4, 2015. **(A)** Whole fruit and flower showing the cuticle is only present on the fruit epidermis, scale bar: 200 µm. **(B)** Fragmented cuticle, scale bar: 100 µm.

The cuticle was seemingly absent from younger fruit collected in April and May (Figures 2A, B, respectively), as the epidermal cells were readily distinguished from each other and the glossy coat surrounding the fruit was minimal. It was seen developing as early as late June, but the fruit's epidermis became visibly smoother and glossier over time as the cuticle developed from June to August. The cuticle entirely envelops the fruit by the beginning of August (Figure

4), as evidenced by the glossy coating seen on the fruit in Figure 4A. The fractured cuticle was visualized (Figure 4) and was found to be approximately 3 µm thick.

When the fruit was ready for discharge at the end of the season, the seed was visualized under the same parameters as the fruit. The general morphology of the seed is shown in Figure 5.

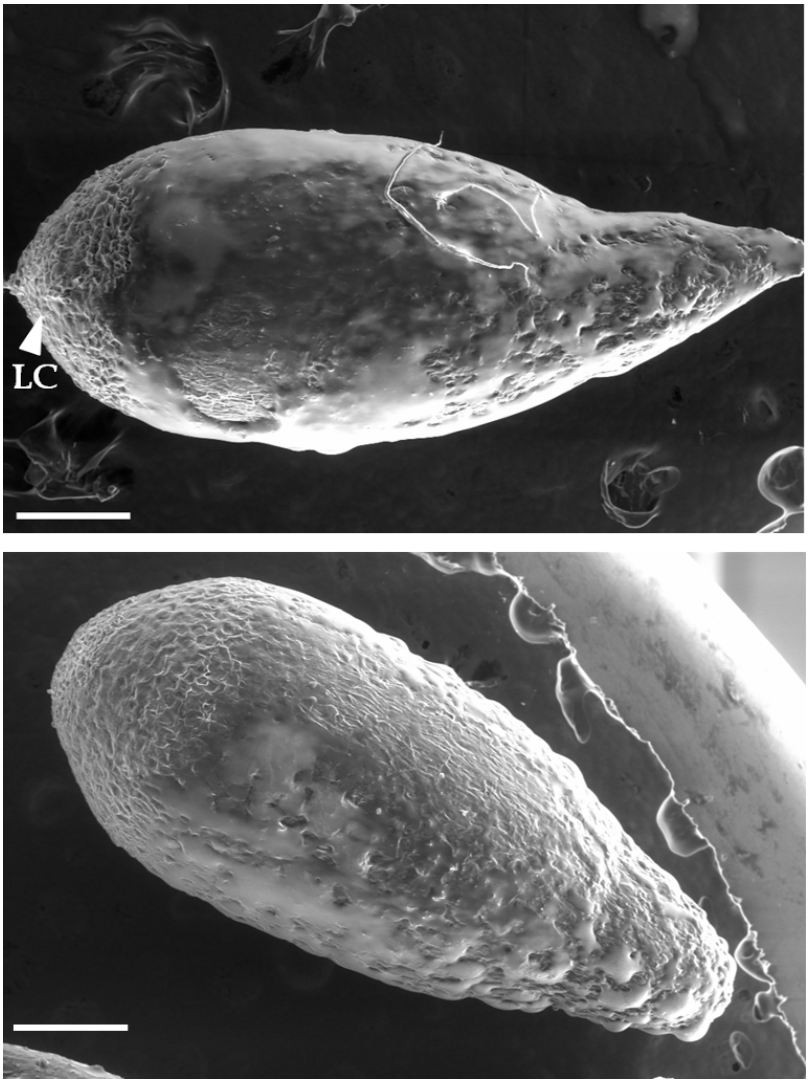


**Figure 5.** SEM micrographs of the mature *A. americanum* seed showing **(A)** lateral and **(B)** side view. Seeds seem to exhibit some lateral compression (LC) and are coated in mucilaginous viscin matrix post-discharge. The external layer is rugged and modified into an adhesive pulp. Scale bar: 400 µm.

Seeds of *A. americanum* resemble teardrops, having a thicker apical end that terminates in a tapered, thinner point (Figure 5). After discharge, seeds retain some of the viscin matrix

from the fruit and possess a notably coarse external coating. Additionally, the seeds seem to exhibit a small degree of lateral compression (Figure 5A). Seeds were found to be approximately 2.7 mm in length and 1.1 mm in diameter at the apical end (n=3). The single seed at maturity is approximately 66% of the length of the entire fruit, and 41% of the total diameter.

## Fruit Growth Measurements

Over the course of the study, fruit length, fruit diameter, stomatal density and the surface area of the tepal whorl were recorded for every fruit collected using ImageJ freeware. Both fruit length and diameter increased over the season in a linear fashion (Figure 6).

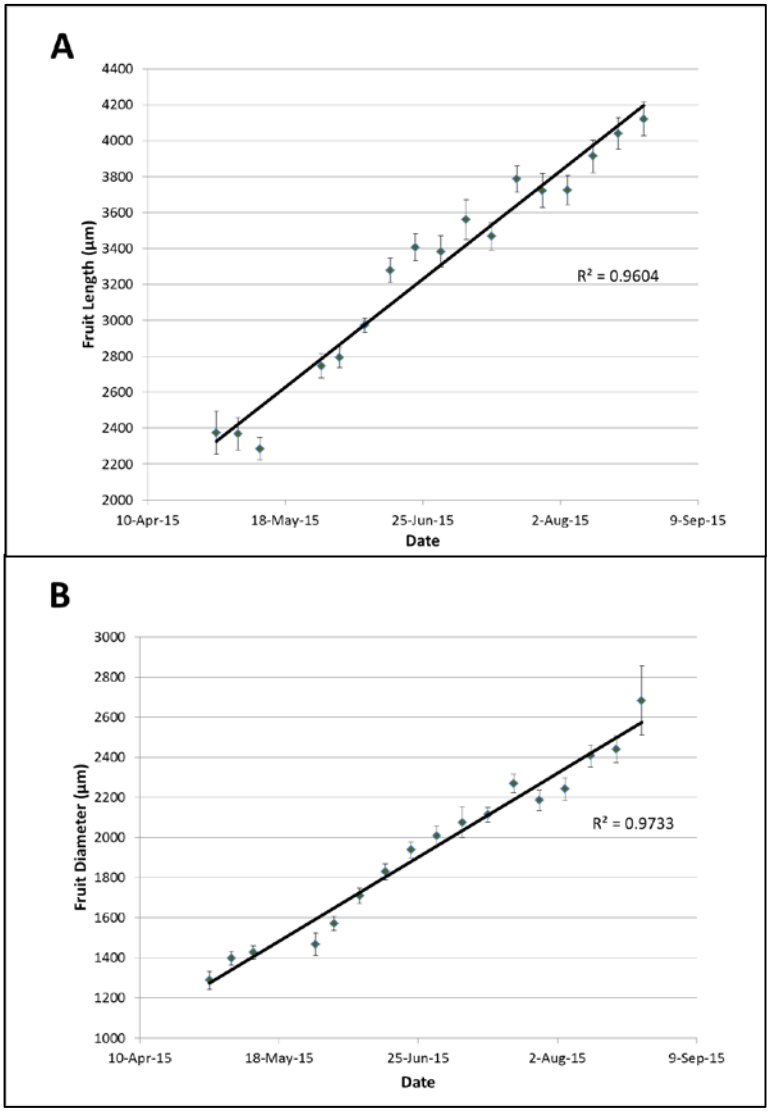


**Figure 6**. Changes in *A. americanum* **(A)** average fruit length and **(B)** average fruit diameter over the sampling period. $R^2 = 0.9604$ (A) and $R^2 = 0.9733$ (B). Equation of the line is y=15.816x+445.84 (A) and y=11.007x - 35.728 (B) where x is the ordinal date.

Second-year fruits of *A. americanum* were found to experience noticeable expansion and elongation over the course of this study (Figure 6). Both fruit length and diameter followed a linear trend and exhibited high $R^2$ values at 0.9604 and 0.9733 respectively, indicating a strong relationship with ordinal date. Equations represent the relationships between fruit length/diameter and ordinal date, respectively. Both fruit length and diameter were found to significantly increase over the growing season (For fruit length and date, $F= 6.24x10^{-12}$, $p = 6.71x10^{-12}$ and for fruit diameter and date, $F= 3.23x10^{-13}$, $p =3.43x10^{-13}$ (Appendix Figures A1 and A2). Lastly, the rate of change in the fruit length was found to be 0.0158 mm/day in the growing season, whereas change in diameter was found to be 0.011 mm/day.

Micrographs of the fruits were also analyzed to deduce stomatal density. As the SEM dehydrates samples within the partial vacuum, all stomata were counted in this study, closed or not, to avoid data being skewed by artefacts produced by the SEM. This leads to a potential overestimation of the natural transpiration rates. The total number of stomata on the tepal whorl did not significantly change over time (data not shown), while the stomatal density followed a polynomial curve with a declining trend over the season (Figure 7).

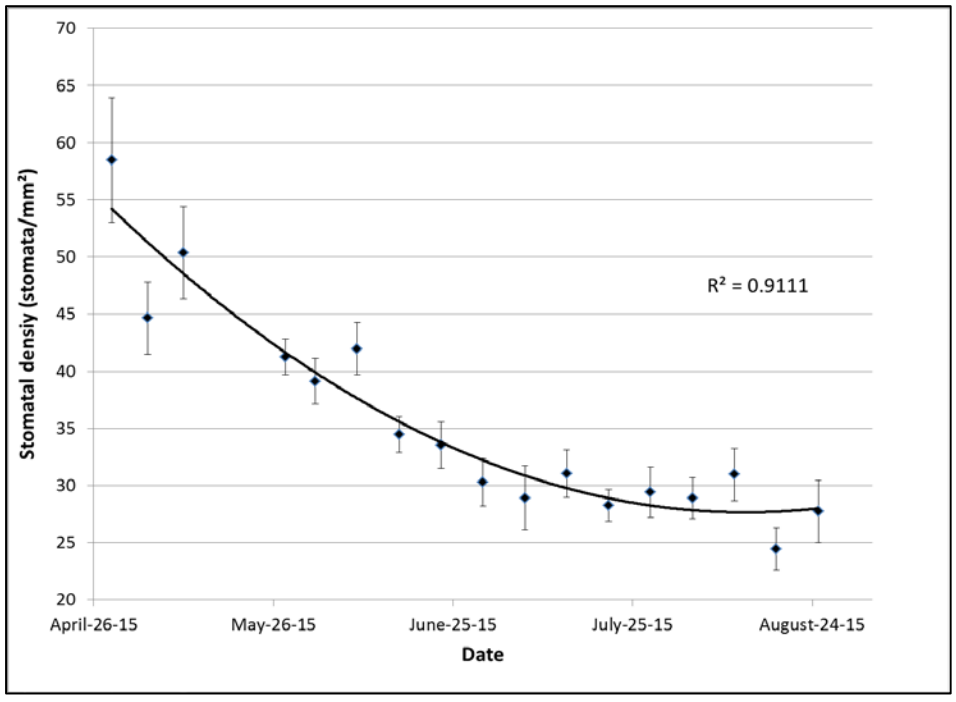


**Figure 7**. Stomatal density (stomata/mm²) of the tepal whorls recorded through the growing season

with standard error in a polynomial relationship. $R^2$=0.9111, line equation is $y = 0.0024x^2 - 200.11x + 4E+06$ where x is the ordinal date. $F = 4.4x10^{-8}$, $p = 1.27x10^{-6}$

Stomatal density was calculated specifically from the tepal whorl, as the fruit was never observed to bear stomata. Stomatal density declines from approximately 51 stomata/$mm^2$ (mean of the first three points) to 28 stomata/$mm^2$ (mean of the last three points), an overall 45% reduction. The data were tested using polynomial regression analysis (Appendix Figure A3), and determined that the change in stomatal density over time is significant ($F =4.4x10^{-8}$, $p = 1.27x10^{-6}$).

# DISCUSSION

## Developmental Observations

*Arceuthobium americanum* has a life cycle involving developmental patterns that are not typical of dicotyledonous flowering plants. *A. americanum* was confirmed to possess transverse stomata (openings arranged perpendicular to the long axis of the plant), which are rare among angiosperms, but characteristic of the Santalaceae family (Butterfass 1987). The exposed floral structures of the DM fruit (the tepals and the stigma) persisted into the second year of fruit growth well after fertilization had already occurred; in typical flowering plants, the petals, sepals, stamens, and the stigma/style degenerate as a result of programmed cell death, while the fruit develops and matures (Wagstaff et al. 2003). In most of these cases, the ovary is retained on the plant without the other floral organs. While it is possible that these structures serve no function in fruit maturation, it may be that the DM fruit requires that floral structures continue to exist into the second year of development for an unidentified reason. As stated previously, the fruit is completely devoid of stomata over the course of this study and as such, the fruit tissue is unable to carry out gas exchange, and thus cannot generate ATP through cellular respiration, without the assistance of the tepal whorl. This offers a possible reason for the persistence of the floral organs through the fruit's maturation period.

The fruit bears a waxy cuticle, found to be approximately 3 µm thick (Figure 4), and has been previously documented (Hawksworth and Wiens 1996). Cuticles in plants provide structural support, protection, and prevent water loss by water proofing the epidermis with a wax coat (commonly constructed from cutin) (Neinhuis and Barthlott 1997). The cuticle that surrounds the fruit likely serves to retain water that is later used to facilitate hydrostatic pressure build-up preceding discharge. The fruit diameter increases noticeably through the second year of development, expanding to be visibly larger than the diameter of the tepal whorl at the end of the season (Figure 2). This change can be likely attributed to the build-up of the fruit's pressure and mass during the differentiation of the pericarp layers. The cuticle, providing a resilient coat around the fruit, could also prevent premature bursting of the fruit and enable it to accumulate internal pressure as a result of water accumulation and cellular growth, but further work examining the cuticle's array of functions in DM is needed.

Over the course of the season, the fruit changes its orientation relative to the vertical axis of the DM stems (Figure 3). Initially, the fruit emerges erect from the bract (i.e., stigma oriented up), but as the pedicel elongates throughout the second year, it recurves so that the fully-mature fruit will point downwards (i.e., stigma oriented down). The abscission layer, which severs and allows the seed to release, is located approximately 80 µm from where the pedicel joins the base of the fruit (Figure 1). This repositioning functions in augmenting the distance and height in which the DM seed can achieve after explosive discharge. To further optimize its trajectory and inoculation success, the seed tapers like a teardrop (Figure 5) to minimize turbulent flow and thus maximize potential distance travelled. The seed also retains a portion of the viscin matrix post-discharge, allowing it to adhere to the host more readily. We also observed the coarse external layer of the seed to be modified into a mucilaginous pulp lacking an integument, as has been observed previously and described as a pseudo-seed coat (Ross Friedman and Sumner 2009).

## Stomata Driving Innovation in Land Plants

The process of transpiration is a major driving force in land plant evolution (Beerling 2005). Transpiration via stomata influences plant physiology by moving water through the plant's tissues, supplying gas exchange for use of $CO_2$ in the Calvin cycle, and cooling the plant's tissues down via water evaporation. Beerling (2005) provides evidence that stomatal density has driven land plant evolution in the past through the cooling mechanism that is part of transpiration. In land plants, it is accepted that a higher stomatal density enabled the evolution of megaphylls (larger leaves with abundant vascular tissue) in vascular plants by providing sufficient temperature regulation to counterbalance the larger surface area. The larger surface area of megaphylls opens them up to the danger of burning in strong sunlight more than the microphylls of lycophytes and bryophytes. The temperature regulation provided by transpiration is believed to be a very active force in land plant evolution (Beerling 2005).

Studies on stomatal density in the floral organs of other plants are limited, but in comparison to other flowers (Azad et al. 2006), the density of stomata in the tepal whorl of DM just before the fruit discharges is relatively low. I have provided preliminary evidence that the stomatal density in *Arceuthobium americanum* declines significantly throughout the growing season to reach that relatively low density (Figure 7). It was found that the stomata number on

the tepal whorl does not significantly change over time (data not shown). It is therefore possible that this decline could be coincidental, resulting simply from an increase in surface area with a constant stomata number, and thus is simply a passive consequence of its growth. However, stomata have a profound influence on plant physiology, and thus this decline may play a role in *A. americanum's* development; the decline might even be accelerated as a result of active reduction in stomatal number as the fruit matures, likely through dedifferentiation of the guard cells into meristemoid mother cells (Hall et al. 1996), but as stomatal number was not found to decrease significantly over time in this study, the reduction is likely passive. Previous work has shown that *Viscum album,* European mistletoe, which is closely related to *A. americanum,* in fact does not close its stomata due to insensitivity to abscisic acid (Escher et al. 2008), and thus it is highly likely that *A. americanum* similarly does not close its stomata. As a result, the stomatal density calculated in this work (counting open and closed stomata) could accurately reflect a decline in transpiration. In exploring the functionality and influence of stomata in both plant evolution and development, possible explanations can be offered as to why the stomatal density declines, but the mechanism for decline (passive, active, or a combination) will need to be explored in the future.

## Stomata in Water Retention

Plants open and close their stomata in response to many environmental stimuli, including sunlight intensity, $CO_2$ requirements, accumulation of $O_2$ within the plant, and water availability (Rizhsky et al. 2002; Wilkinson and Davies 2002; Engineer et al. 2016). Transpiration involves a constant trade-off for plants as it causes water loss in order to allow $CO_2/O_2$ gas exchange for photosynthesis. By closing their stomata, transpiration rates decline and the plant can more effectively retain water in its tissues, but at the expense of gas exchange. As *A. americanum* has a functionally limitless water source from its host, it is logical to hypothesize that the stomatal density would reflect an optimization of gas exchange as there would be no concomitant water loss. One would expect many or an increasing number of stomata, or stomata that do not close in DM. However, *A. americanum* shows the opposite of what is expected: a decline in stomatal density as the fruit develops.

A possible explanation for this decline in stomatal density lies in the need for water to accumulate in order to build up hydrostatic pressure within the fruit prior to discharge (Ross

Friedman et al. 2010). As mentioned previously, the mesocarp undergoes differentiation into viscin and vesicular cells in the second year of growth (Kelly et al. 2009). Viscin cells are an elongated cell type with a high concentration of hydrophilic carbohydrates (Ross Friedman and Sumner 2009); they secrete viscin, a mucilaginous sugar matrix, via Golgi bodies. While the hygroscopic viscin accumulates water, the hydrophobic layer of vesicular cells, encircling the viscin, is thought to prevent this moisture from exiting the fruit. As transpiration through the stomata is involved in the loss of water, the decrease in stomatal density observed in DM could be an adaptation to maximize hydrostatic pressure prior to discharge by minimizing water loss. This method of water retention could be working in concert with type 2 plasma membrane intrinsic protein (PIP2) aquaporins, which have been shown to be present in the viscin of young fruits (Ross Friedman et al. 2010), but become less numerous as the fruit matures. Furthermore, the expression of PIP2 aquaporins in mature fruits has been found to be noticeably less than in the immature fruits (Urban, unpublished data). Importantly, mesocarp differentiation into viscin and vesicular cells is strongly evident by the beginning of August (Kelly et al. 2009), which is approximately when the stomatal density was found to level off (Figure 7). This could indicate that at this point in the plant's growth, internal differentiation has been completed and the decline in stomatal density acts to help retain accumulated water. From then until discharge, it is likely that the fruit undergoes cellular growth until it is physiologically ready to discharge (as is shown in the linear relationships in Figure 6), but the details of the criteria for explosive seed discharge are still unclear.

Hydrostatic pressure is an integral part of the fruit's explosive discharge and it is likely established and maintained by the following factors: (1) the vesicular layer waterproofing the inside of the fruit (Kelly et al. 2009), (2) the formation of the cuticle on the fruit body (Hawksworth and Wiens 1996), (3) the down regulation of an integral aquaporin prior to discharge (Ross Friedman et al. 2010; Urban, unpublished data), and (4) a decline in stomatal density leading up to discharge, which prevents water loss from the fruit.

## Stomata in Temperature Regulation

The advent of higher stomatal densities is believed to be a major contributing factor to the evolution of land plants: evading burns from strong sunlight as the transpired water would cool the megaphylls (Beerling 2005). Knowing that this form of cooling is influential enough to

drive the evolution of such a widespread plant structure today (megaphylls) indicates that stomatal density acts as a strong adaptive trait in temperature regulation.

Given the impact that stomatal density has on temperature regulation, another explanation, which may or may not be correlated with water retention, can be offered for how stomata contribute to explosive discharge. Hinds et al. (1963) explored the dispersal mechanism of DM and found that heating the mature fruit triggers its explosive discharge. In recent years, deBruyn and colleagues (2015) demonstrated that the fruit is capable of thermogenesis, which facilitates this discharge process. By reducing the number of stomata, the cooling mechanism normally provided by stomata could work in reverse; in DM, a reduced rate of transpiration could actually increase the fruit's internal temperature, which could aid in the fruit's explosive discharge. The reduced transpiration rate may facilitate explosive discharge by working in tandem with the thermogenesis documented by deBruyn et al.(2015) The fruit may be heated to a point where the hydrostatic pressure exerts enough force on the abscission layer where the pedicel joins the fruit (Ross Friedman and Sumner 2009) that it severs, discharging the seed. This is of particular interest when considering that all trees sampled had a stomatal density from 24-27 stomata/mm² by the end of August (Table 1A). The closeness of these values suggests that a threshold of fruit volume-to-transpiration ratio must be achieved before the fruit can successfully discharge. If a lack of transpiration is an important factor in explosive discharge, then the sunlight striking the fruit must be sufficient to push it past this hypothetical threshold. Exogenous heat may be required for this process due to the wasteful nature of thermogenesis in plants, in which a proton motive force in the mitochondria is harnessed to produce heat rather than ATP through a different route in the mitochondrial electron transport chain (ETC) (Zhu et al. 2011). This divergence in the ETC is energetically inefficient, and thus *A. americanum* could potentially supplement the heat required for discharge by harnessing exogenous heat from the sun through a reduction in transpiration. This hypothesis draws on the knowledge that heating the fruit leads to its discharge (Hinds et al. 1963), and the DM has likely developed a way to orchestrate this process.

## Future Work

In exploring the development of *A. americanum,* our objectives have been met in evaluating 1) the persistence of floral organs, 2) the changes in fruit diameter/length and stomatal

density, and 3) the qualitative developmental trends in second year fruits preceding discharge. This study has demonstrated that during the second year of fruit maturation of female *A. americanum*, fruit length and diameter both experience significant increases over the growing season, while stomatal density declines significantly. At this time, the information needed to draw concrete conclusions about the physiological and developmental changes that facilitate DM's highly unique seed dispersal is lacking. However, given that the results of this study and others (Kelly et al. 2009; Urban, unpublished data) suggest that water accumulation followed by retention to develop a sufficient amount of hydrostatic pressure is key in enabling the explosive discharge, future work in elucidating this process should focus on water movement and physiology inside the fruit. To further our knowledge about the influence of the stomata on this process, transpiration rates leading up to explosive discharge should be studied. As the SEM dehydrates samples within the partial vacuum, all stomata were counted in this study, closed or not. This method leads to an overestimation of the natural transpiration rates, as plants regulate stomatal apertures tightly in response to environmental cues. For this reason, research looking specifically at stomatal openings on the flower would provide a more accurate representation of the transpiration rates that are actually occurring. This type of study could be done by taking sections of the tepals and examining them under a light microscope, which would avoid the dehydration that occurs within the SEM. Furthermore, the fruit's internal temperature relative to sunlight intensity may be important in articulating the discharge process. Although it has been established that the fruit undergoes a form of thermogenesis (deBruyn et al. 2015), environmental temperature influence on the discharge has not been critically evaluated. The results presented here provide preliminary evidence supporting the idea that stomata influence the fruit development and explosive discharge in some way, and further studies must focus on what processes they are facilitating that contributes to these events, and deducing if they are a causative factor rather than simply correlative.

## Conclusion

The story of DM explosive discharge has been slowly building, and the entire process is now coming into view. This project has contributed to this body of knowledge by providing definitive evidence of declining stomatal density and continuous fruit growth, which suggests a great influence on both the internal temperature and transpiration rates. Together with aspects of

the fruit's biology that have already been articulated, such as mesocarp differentiation (Kelly et al. 2009), down regulation of aquaporins (Ross Friedman et al. 2010), and thermogenesis (deBruyn et al. 2015), these results indicate that the biological preparation for discharge is as much an internal process as it is an external one. In this case, it is clear that *A. americanum* has developed a plethora of methods to optimize its explosive discharge.

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# APPENDIX

| SUMMARY OUTPUT | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| *Regression Statistics* | | | | | | | | |
| Multiple R | 0.980009868 | | | | | | | |
| R Square | 0.960419341 | | | | | | | |
| Adjusted R Square | 0.957780631 | | | | | | | |
| Standard Error | 123.0048014 | | | | | | | |
| Observations | 17 | | | | | | | |
| | | | | | | | | |
| ANOVA | | | | | | | | |
| | *df* | *SS* | *MS* | *F* | *Significance F* | | | |
| Regression | 1 | 5506977.037 | 5506977.037 | 363.9729741 | 6.24463E-12 | | | |
| Residual | 15 | 226952.7175 | 15130.18117 | | | | | |
| Total | 16 | 5733929.755 | | | | | | |
| | | | | | | | | |
| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
| Intercept | -663909.0283 | 34972.09175 | -18.98396679 | 6.70679E-12 | -738450.2774 | -589367.7792 | -738450.2774 | -589367.7792 |
| X Variable 1 | 15.81646675 | 0.829038891 | 19.07807574 | 6.24463E-12 | 14.04941218 | 17.58352132 | 14.04941218 | 17.58352132 |

**Figure A1**. Linear regression output of fruit length over time at a 95% confidence interval.

| SUMMARY OUTPUT | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| *Regression Statistics* | | | | | | | | |
| Multiple R | 0.985114787 | | | | | | | |
| R Square | 0.970451143 | | | | | | | |
| Adjusted R Square | 0.968481219 | | | | | | | |
| Standard Error | 0.073210483 | | | | | | | |
| Observations | 17 | | | | | | | |
| | | | | | | | | |
| ANOVA | | | | | | | | |
| | *df* | *SS* | *MS* | *F* | *Significance F* | | | |
| Regression | 1 | 2.640406531 | 2.640406531 | 492.6338513 | 6.94153E-13 | | | |
| Residual | 15 | 0.080396623 | 0.005359775 | | | | | |
| Total | 16 | 2.720803154 | | | | | | |
| | | | | | | | | |
| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
| Intercept | -0.025774661 | 0.090518083 | -0.284745984 | 0.779732371 | -0.218709388 | 0.167160067 | -0.218709388 | 0.167160067 |
| X Variable 1 | 0.010951869 | 0.000493431 | 22.19535652 | 6.94153E-13 | 0.009900146 | 0.012003591 | 0.009900146 | 0.012003591 |

**Figure A2**. Linear regression output of fruit diameter over time at a 95% confidence interval.

| SUMMARY OUTPUT | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| *Regression Statistics* | | | | | | | | |
| Multiple R | 0.986567041 | | | | | | | |
| R Square | 0.973314526 | | | | | | | |
| Adjusted R Square | 0.971535494 | | | | | | | |
| Standard Error | 69.81709646 | | | | | | | |
| Observations | 17 | | | | | | | |
| | | | | | | | | |
| ANOVA | | | | | | | | |
| | *df* | *SS* | *MS* | *F* | *Significance F* | | | |
| Regression | 1 | 2666816.317 | 2666816 | 547.1035549 | 3.22774E-13 | | | |
| Residual | 15 | 73116.40436 | 4874.427 | | | | | |
| Total | 16 | 2739932.721 | | | | | | |
| | | | | | | | | |
| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
| Intercept | -462352.9179 | 19850.03736 | -23.2923 | 3.432E-13 | -504662.2711 | -420043.5648 | -504662.2711 | -420043.5648 |
| X Variable 1 | 11.0065039 | 0.470559584 | 23.39024 | 3.22774E-13 | 10.00352989 | 12.00947791 | 10.00352989 | 12.00947791 |

**Figure A3**. Polynomial regression output of stomatal density over time at a 95% confidence interval.

**Table A1**. Raw data means of fruit length, fruit diameter and stomatal density

| Date | Fruit Length (µm) | Fruit diameter (µm) | Stomatal Density (stomata/mm²) |
|---|---|---|---|
| April 16 2015 | * | * | 39.23 |
| April 29 2015 | 2374.86 | 1287.49 | 58.47 |
| May 5 2015 | 2367.22 | 1398.36 | 44.66 |
| May 11 2015 | 2285.54 | 1426.12 | 50.37 |
| May 28 2015 | 2746.63 | 1466.77 | 41.25 |
| June 2 2015 | 2793.85 | 1570.90 | 39.13 |
| June 9 2015 | 2972.49 | 1710.12 | 41.96 |
| June 16 2015 | 3277.34 | 1830.02 | 34.46 |
| June 23 2015 | 3406.85 | 1939.15 | 33.54 |
| June 30 2015 | 3383.59 | 2007.70 | 30.28 |
| July 7 2015 | 3560.47 | 2075.25 | 28.92 |
| July 14 2015 | 3467.22 | 2113.64 | 31.06 |
| July 21 2015 | 3786.76 | 2268.35 | 28.26 |
| July 28 2015 | 3722.44 | 2185.26 | 29.42 |
| August 4 2015 | 3725.33 | 2241.35 | 28.89 |
| August 11 2015 | 3913.35 | 2406.99 | 30.96 |
| August 18 2015 | 4041.06 | 2440.22 | 24.43 |
| August 25 2015 | 4121.06 | 2682.82 | 27.73 |

* - Denotes missing data